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Institute of Nutraceutical Research Pty Ltd

PROVISIONAL SPECIFICATION

Invention Title:

Connective tissue derived polypeptides

The invention is described in the following statement:

Technical Field

The present invention relates to novel methods for preparing and recovering connective tissue derived polypeptides, their uses in methods of treatment, and protection of connective tissues in arthritis and other degenerative diseases.

Background Art

Diseases of the musculoskeletal system such as rheumatoid arthritis (RA), osteoarthritis (OA), disc degeneration (DD), and osteoporosis (OP) are a major cause of morbidity throughout the world. These diseases have a substantial influence on health and quality of life and inflict an enormous cost on health systems.

The aetiology of OA is considered to be multi-factorial with ageing, mechanical, hormonal and genetic factors all contributing to varying degrees. OA emerges as a clinical syndrome when these etiological determinants result in sufficient joint tissue damage to cause synovial inflammation and the appearance of the symptoms of pain and impairment of function.

RA is thought to arise as a consequence of extrinsic and/or intrinsic triggering of an autoimmune response in genetically susceptible individuals. The aggressive inflammation initiated in the joints of RA patients by the activation of their immune system is manifest by the release of pro-inflammatory cytokines, proteinases and free radicals. All of these mediators have the ability to promote the destruction of cartilage, bone and other intra-articular joint tissues leading to further impairment of joint function and progression of the disease. While both OA and RA show common pathological features of cartilage destruction and synovial inflammation, the origins and temporal history of these events are clearly distinct. Nevertheless, destruction of joint cartilage is common to both OA and RA and there is now strong evidence that the breakdown and release of the matrix components from cartilage play a significant role in the chronicity of these diseases.

Cartilage may be considered as an anisotropic biomaterial composed essentially of a three-dimensional fibrous network of type II collagen fibrils copolymerised with types IX and XI collagens. Type II collagen accounts for over 90% of the total collagen of adult cartilage while the type IX content is only 1-2%. Although only a minor component of cartilage, type IX collagen provides an important role in maintaining the type II collagen fibrous network assembly which is essential for the optimum physical function of this weight-bearing tissue. Type IX collagen resides on the surface of the type II collagen fibrils to which it is covalently linked via, at least 2 trivalent pyridinoline cross-links, particularly at sites where the type II fibril network

intersects. By cross-linking the type II collagen fibrils type IX collagen would appear to constrain the expansion caused by the imbibition of water molecules attracted into the tissue by the trapped negatively charged proteoglycan (PG) aggregates (referred to herein as aggrecans).

The aggrecans of cartilage are macro-molecular aggregates of PG subunits which are non-covalently attached along the length of a hyaluronic acid chain. Each aggrecane may contain 20-50 PG subunits and their interaction with the HA backbone is stabilised by ternary interactions with link protein. The PG subunits consist of a protein core to which up to 100 GAG chains are covalently linked. The major GAG substituents of the PGs are the chondroitin sulfates (ChS) and keratan sulfate.

In addition to the collagens and proteoglycans, cartilage also contains a large number of non-collagenous proteins, the most abundant being cartilage oligomeric protein (COMP), cartilage matrix protein (CMP), and thrombospondin. COMP is considered to be a key structural component of the cartilage matrix since it interacts with type IX collagen and plays a role in the development and assembly of type II collagen fibrils. In the early stages of arthritis when cartilage breakdown is increased, PGs, type IX collagen and COMP fragments are some of the first matrix components to be released into synovial fluid by the action of endogenous proteinases. These products of cartilage breakdown have been shown to be antigenic and can induce an inflammatory response within arthritic joints and exacerbate the existing condition.

The autoantigen driven synovitis produced by the above reactions, once established within affected joints, can alter the metabolism of resident synoviocites, the major cellular source of synovial HA in joints. Inflammatory mediators released from local tissue macrophage and infiltrating leucocytes can also promote increased vascular permeability and the dilution of synovial fluid by plasma fluid, thereby decreasing local HA concentration. This dilution of HA coupled with a reduction in its molecular size due to abnormal synthesis by synoviocites results in a substantial decrease in the rheological properties of synovial fluid (SF) and consequently its ability to lubricate and protect articular cartilage.

In arthritic diseases, the excessive breakdown of cartilage and bone and the concomitant elicitation of an inflammatory reaction provoked by the release of autoantigens are responsible for their chronicity. However, there is accumulating evidence to indicate that these matrix molecules could also be responsible for the initiation of joint diseases. Indeed, systemic administration of adjuvants together with type II collagen or other matrix components to laboratory animals have been used to produce animal models of arthritic disease.

In the case of cartilage collagens, this knowledge has led to the development of means of treating rheumatic diseases by using the concept of oral tolerisation. Thus it has been shown that suppression of type II collagen-induced arthritis in animal models can be achieved by oral administration of low doses of type II collagen. Oral tolerisation of arthritic patients by administration of type II collagen has also been shown to be effective clinically and therapeutic effects have been reported with small (less than 100mg) daily doses of type II collagen antigens. Studies using rheumatoid and juvenile rheumatoid arthritis patients confirmed the efficacy and safety of low daily oral doses of type II collagen derived from chick sterna (Barnett *et al*, A pilot study of oral type II collagen in the treatment of juvenile rheumatoid arthritis. Arthritis. Rheum. 39:623-628, 1996). Other studies have indicated that cartilage collagens obtained from bovine sources were less effective than those prepared from chick cartilage.

Unfortunately, some studies have found that high doses of type II collagen may in fact exacerbate disease. With regard to other cartilage collagens (eg. IX, X, XI), at least one study has indicated that type IX collagen is not as effective as type II collagen in some animal arthritis models (Lu S *et al*, Different therapeutic and bystander effects by intranasal administration of homologous type II and type IX collagens on the collagen- induced arthritis and Pristane-induced arthritis in rats. Clin Immunol. 90:119-127, 1999).

Furthermore some difficulty has been observed in the production of commercial quantities of pure cartilage collagens. Most recently, the full length alpha subunit chains of IX collagen have been recombinantly produced. (Myers L K . *et al*, Immunogenicity of recombinant Type IX collagen in murine collagen-induced arthritis. Arthritis Rheum. 46:1086-1093, 2002). The recombinantly produced full-length protein has been tested for immunogenicity in murine models and it was found that Type IX collagen was able to ameliorate the disease, however, in contrast to Type II collagen, it was unable to induce overt arthritis in mice immunized with this protein. In an earlier publication the same group described similar experiments using Type IX collagen obtained by limited digestion of chick, human, rat or bovine foetal cartilage with pepsin.

Importantly, pepsin which is not present in cartilage and is not known to cleave collagens in the non-helical NH2 domains. With regard to the manufacture of matrix component from connective tissue, most of the proteolytic enzymes used for their digestion and release are derived from bacterial, plant or bovine offal sources because of their broad range of substrate specificity and commercial availability. The amino acid sequences which are recognised and cleaved by these various enzymes as well as

the amino acid sequences of the polypeptide fragments generated by their proteolytic actions, are therefore quite different to the sites of cleavage and polypeptide sequences produced by the endogenous proteinases within the native mammalian connective tissues.

Summary of Invention

The present invention provides methods for recovering polypeptides from connective tissue wherein connective tissue particles are subjected to autolysis in the presence of an autolysis medium such that a mixture of glycosaminoglycan peptides and polypeptides are released from the connective tissue particles into the autolysis medium. According to the invention, polypeptides are recovered from the medium and are separated according to size and charge. The invention also provides methods for separating and identifying the recovered polypeptides. The process of inducing autolysis has previously been described in PCT/AU03/00061 (in the name of the Applicant), which is incorporated herein by reference.

Accordingly in a first aspect the present invention provides a method for preparing a polypeptide having anti-arthritic or anti-inflammatory activity, the method comprising isolating a mixture comprising a GAG-peptide and a polypeptide having a molecular weight of greater than 10,000Da by autolysis from connective tissue, separating the GAG-peptide from the polypeptide, and recovering the polypeptide.

In a related aspect, the present invention provides a method for preparing a polypeptide having anti-arthritic or anti-inflammatory activity, the method comprising

(i) incubating a connective tissue in an autolysis medium that provides a buffered pH range of between about pH 2.5 and about pH 8.5 for a time and under conditions sufficient to release at least one GAG-peptide and at least one polypeptide having a molecular weight of greater than about 10,000Da,

(ii) recovering a mixture comprising a GAG-peptide and a polypeptide from the autolysis medium;

(iii) separating the polypeptide from the GAG-peptide; and

(iv) recovering the polypeptide having a molecular weight of greater than about 10,000Da.

Any well known separation techniques such as for example, chromatography, ion exchange techniques, gel filtration (eg. diafiltration or ultrafiltration), gel electrophoresis (eg. one dimensional or two dimensional) or any other method of separating polypeptides according to their size and molecular weight, or combination thereof, can be used to recover the polypeptide from the mixture.

A combination of the same or similar separation techniques may be used and may be repeated. 'In this way, fractions of polypeptides of different molecular weight ranges may be obtained and individual polypeptides can also be recovered.

The inventors have further analysed and identified the individual polypeptides separated by the method of the present invention.

Polypeptides recovered by the method of the invention are connective tissue derived polypeptides. The inventors have found that the recovered polypeptides and mixtures thereof have enhanced and/or different pharmacological activities to the GAG-peptide/polypeptide mixtures. The polypeptides according to the present invention are those connective-tissue derived polypeptides having anti-arthritic or anti-inflammatory activity. In one embodiment, polypeptides of the invention and mixtures thereof reduce rear paw inflammation in rats with collagen induced arthritis. In another embodiment, polypeptides of the invention and mixtures thereof decrease tail inflammation in rats with collagen induced arthritis. In another embodiment, polypeptides decrease fore paw inflammation in rats with collagen induced arthritis. In yet another embodiment, polypeptides of the invention and mixtures thereof decrease weight loss in rats with collagen induced arthritis.

Accordingly, in a second aspect the present invention provides a connective tissue derived polypeptide, obtainable by the method of the invention, having anti-arthritic or anti-inflammatory activity.

The effects observed for the polypeptides and mixtures thereof on rats with collagen induced arthritis also provide application for the polypeptides, and mixtures thereof, in the treatment, protection and restoration of connective tissues in inflammatory and degenerative tissue diseases such as rheumatoid arthritis and osteoarthritis. Preferably, the polypeptides of the present invention prevent antigen driven autoimmune diseases. In another preferred embodiment, polypeptides of the invention and mixtures thereof reduce symptoms associated with antigen driven autoimmune diseases.

A third aspect of the invention provides for the use of one or more connective tissue derived polypeptide having anti-arthritic or anti-inflammatory, in the preparation of a medicament for the treatment or prevention of autoimmune disease in a subject, wherein said polypeptide is obtainable by the methods of the first aspect of the present invention.

In a related aspect the invention provides for the use of one or more connective tissue derived polypeptide having anti-arthritic or anti-inflammatory, in the preparation of a medicament for the treatment or prevention of autoimmune disease in a subject,

wherein said polypeptide is a polypeptide according to the second aspect of the invention.

In a fourth aspect the invention provides a prophylactic or therapeutic vaccine composition for eliciting an immune response against connective tissue derived polypeptides, said composition comprising a connective tissue derived polypeptide or an fragment thereof in combination with a pharmaceutically acceptable carrier, excipient, diluent and/or adjuvant.

Preferably, each polypeptide or fragment thereof is formulated with a suitable adjuvant to protect a subject from developing an connective tissue disease.

In a fifth aspect the invention provides a method of treating or preventing a autoimmune disease in a subject comprising administering to the subject an amount of a pharmaceutically acceptable composition comprising a connective tissue derived polypeptide having anti-arthritic or anti-inflammatory wherein said polypeptide is obtainable by the methods of the first aspect of the invention.

In a related aspect the invention provides a method of treating or preventing a autoimmune disease in a subject comprising administering to the subject an amount of a pharmaceutically acceptable composition comprising a connective tissue derived polypeptide having anti-arthritic or anti-inflammatory wherein said polypeptide is a polypeptide according to the second aspect of the invention.

In a sixth aspect, the invention provides a method of inducing cartilage formation in an individual, comprising administering to the individual an effective amount of a connective tissue derived polypeptide having anti-arthritic or anti-inflammatory, wherein said polypeptide is obtainable by the methods of the first aspect of the invention. In a related embodiment the polypeptide is a polypeptide according to the second aspect of the invention.

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

Detailed Description of the Figures

Figure 1 is a schema employed for the separation and sub-fractionation of peptacans into purified GAG-peptides and polypeptides using ion exchange media.

Figure 2 is a photographic representation of separation of polypeptides by SDS-PAGE.

Figure 3 is a photographic representation of SDS-PAGE 2-D electrophoresis of polypeptides.

Figure 4 identifies the peptides separated by the SDS-PAGE 2-D electrophoresis after tryptic digestion and Matrix Assisted Laser Desorption Ionisation (MALDI) mass spectrometry of the cleaved fragments determined by comparison with corresponding trypsin digestion fragments available in published databases.

Figure 5 is an amino acid sequence of COMP protein as listed in the database of the National Center for Biotechnology Information (NCBI) of the National Institutes of Health, Bethesda, Maryland, United States of America under NCBI Accession No NP_511040.

Figure 6 is an amino acid sequence of Type IX collagen alpha 1 as listed in the database of the National Center for Biotechnology Information (NCBI) of the National Institutes of Health, Bethesda, Maryland, United States of America under NCBI Accession No NP_511040.

Figure 7 is a table of the physical characteristics (MW and pI) of protein fragments found in Calcium Peptacan after ion-exchange treatment and 2D electrophoresis (Table 1).

Figure 8 is a table of results for rat CIA-prophylactic treatment.

Figure 9 is a table of results for rat CIA therapeutic treatment.

Figure 10 is a table of results for gastrotoxicity in rat CIA .

Detailed Description of the Invention

In a first aspect the present invention provides a method for preparing a polypeptide having anti-arthritic or anti-inflammatory, the method comprising isolating a mixture comprising a GAG-peptide and a polypeptide having a molecular weight of greater than 10,000Da by autolysis from connective tissue, separating the GAG-peptide from the polypeptide; and recovering the polypeptide.

In a related aspect, the present invention provides a method for preparing a polypeptide having anti-arthritic or anti-inflammatory , the method comprising

(i) incubate a connective tissue in an autolysis medium that provides a buffered pH range of between about pH 2.5 and about pH 8.5 for a time and under conditions sufficient to release at least one GAG-peptide and at least one polypeptide having a molecular weight of greater than about 10,000Da,

(ii) recovering a mixture comprising a GAG-peptide and a polypeptide from the autolysis medium;

(iii) separating the polypeptide from the GAG-peptide; and

(iv) recovering the polypeptide having a molecular weight of greater than about 10,000Da.

Autolysis

Connective tissue is an animal tissue that supports organs, fills the spaces between them, and forms tendons and ligaments. The term "tissue" as used herein refers to a group of similarly specialised cells that perform a common function. As used herein, tissue is intended to include an organ composed of a given tissue and to the cells, individually or collectively, that compose the tissue.

In one embodiment, the connective tissue is a cartilage. In another embodiment, the connective tissue is non-cartilage material eg. lung, skin, bone, ligament or tendon.

Preferably the cartilage is tracheal, articular, auricular, nasal, sternal, rib skeletal, or antler cartilage. Cartilage may, however, be any type of cartilage.

Connective tissue may be obtained from any animal having connective tissue.

In one embodiment, connective tissue is selected from any one of the following: human, bovine, ovine, porcine, equine, avian, cervine and piscine species. Preferably the connective tissue is bovine, ovine, porcine, cervine, shark or equine.

In one embodiment tissues from young animals are preferable eg a calf. In an alternate embodiment a more mature animal is preferred.

The connective tissue may be treated and washed as required by methods known in the art to remove any adhering soft tissues. In one embodiment the connective tissue is reduced to a particle size. In an alternate embodiment the connective tissue is not reduced to a particle size.

The connective tissue can be reduced to a particle size by means including, but not limited to, mincing, dicing, grinding and the like. In one embodiment particle diameter is less than about 5mm, preferably less than about 4mm, more preferably less than about 3mm. Most preferably, the particle diameter is about 0.1mm to about 3mm.

The terms "incubate" or "incubating" mean to maintain (a chemical or biochemical system) under specific conditions in order to promote a particular reaction.

As used herein the term "autolysis" refers to the digestion of cellular components by endogenous hydrolases and proteinases released from lysosomes or associated with the cell and its pericellular matrix following cell death, causing self digestion of tissue. A person skilled in the art will appreciate that the rate of autolysis will vary with many factors including pH, temperature, concentration, tissue type, tissue particle size and time of incubation.

The term "buffer" refers to a compound, usually a salt, which, when dissolved in an aqueous medium serves to maintain the free hydrogen ion concentration of the solution within a certain pH range, when hydrogen ions are added or removed from the solution. A salt or solution is said to have a "buffering capacity" or to buffer the solution over such a range, when it provides this function. Generally a buffer will have adequate buffering capacity over a range that is within ± 1pH unit of its pK.

The term "about" as used in the context of the present invention means approximately or nearly. In the context of numerical values, without restricting to a strict numerical definition, the term may be construed to estimate a value that is ± 10% of the value or range recited.

In one embodiment the salt is a monovalent salt. Preferably the monovalent salt is selected from any one or more of hydrogen, sodium, potassium, or ammonium. In an alternate embodiment the salt is not a monovalent salt . In another embodiment the salt is a divalent salt selected from any one or more of calcium, magnesium, copper, or zinc. Most preferably the salt is calcium or magnesium.

In one embodiment the pH is in the range of about 2.5 to about 8.5, preferably about 3.5 to about 8.0, more preferably about 4 to about 7 and most preferably about 4.5 to about 7.

The term "condition" refers to other factors which affect the rate, efficiency and amount of autolysis, such as, for example, temperature and time.

In one embodiment the temperature conditions for carrying out the step of autolysis is in the range of from about 20°C to about 45°C, preferably about 25°C to about 45°C, more preferably about 32°C to about 45°C, more preferably about 32°C to about 40°C most preferably about 37°C.

In one embodiment, the autolysis takes up to 48 hours, preferably up to 36 hours, preferably up to 24, preferably up to 16 hours, more preferably 16-24 hours.

In a preferred embodiment, cartilage particles of size 1-3mm are subject to autolysis in an aqueous medium at a pH of 4-5 and temperature of 32-45°C for 16-24 hours.

Glycosaminoglycan (GAG) refers to the polysaccharide chains of proteoglycans, which are composed of repeating disaccharide units containing a derivative of an amino sugar (either glucosamine or galactosamine) glycosidically linked to glucuronic or iduronic acid. The most common derivatives being o-sulfated esters substituted in the 4 or 6 positions of the N-acetylated glucosamine or galactosamine rings.

Examples of GAGs include hyaluronic acid (hyaluronan) (which is non-sulfated), chondroitin sulfate, keratan sulfate and heparan sulfate.

The terms "protein", "polypeptide", or "peptide", when used herein refer to amino acids in a polymeric form of any length.

GAG-peptides and polypeptides can be recovered from the autolysis medium with well known methods. For example, in one embodiment, the residual tissue particles are removed by filtration from the autolysis media and the mixture of GAG-peptide complexes and polypeptides recovered from the supernatant. In another embodiment, the residual tissue particles are not removed from the autolysis medium.

In one embodiment, the supernatant is neutralised by addition of an alkaline solution containing a cation.

In one embodiment the supernatant is freeze dried. In an alternate embodiment the supernatent is not freeze dried.

Alternatively the mixture of GAG-peptide and polypeptide is recovered from the autolysis medium or supernatant by precipitation with excess quantities of acetone, or aliphatic alcohols, such as, for example, ethanol or methanol. In another embodiment, the mixture of GAG-peptide and polypeptide is recovered from the autolysis medium or supernatant by the formation of water insoluble complexes with quaternary ammonium salts such as cetyl pyridinium, chloride. In another embodiment the mixture of GAG-peptide and polypeptide are recovered from the autolysis medium or supernatant by separation using size exclusion or ion-exchange or other forms of column chromatography or membrane filtration technology.

Separating and recovering the polypeptides of the invention

Following recovery of the mixture of GAG-peptide and polypeptide, the polypeptide and GAG-pepetide can be separated by methods well known in the art.

Preferably, the mixture of the GAG-peptide and the polypeptide is subjected to an ion exchange technique. In one embodiment the mixture of the GAG-peptide and

the polypeptide is subjected to ion exchange solid phase media. In one embodiment the solid phase media is DEAE sepharose. In a preferred embodiment, the solid phase media is pre-swollen DEAE-Sepharose-6B.

Recovery of the polypeptide refers to any well known separation technique. In one embodiment the polypeptide can be recovered by, for example, chromatography, ion exchange techniques, gel filtration (eg. diafiltration or ultrafiltration), gel electrophoresis (eg. one dimensional or two dimensional) or any other method of separating polypeptides according to their size and molecular weight, or combination thereof, and which is capable of recovering polypeptides having a molecular weight greater than 10,000Da.

A combination of the same or similar separation techniques may be used and may be repeated. In this way, fractions of polypeptides of different molecular weight ranges may be obtained and individual polypeptides can also be recovered.

The inventors have further analysed and identified the individual polypeptides separated by the method of the present invention.

Accordingly, in one embodiment, the method comprises recovering polypeptides having a molecular weight of greater than about 10,000Da, preferably greater than about 20,000Da, preferably greater than about 30,000Da, preferably greater than about 40,000Da, preferably greater than about 50,000Da, preferably greater than preferably greater than about 60,000Da, preferably greater than about 70,000Da, preferably greater than about 80,000Da, preferably greater than about 90,000Da, or preferably greater than about 100,000Da.

Polypeptides can also be recovered in desired molecular weight ranges. Preferably, the method comprises separating and recovering fractions of polypeptides having a molecular weight of about 10,000Da to about 100,000Da. More, preferably, fractions of polypeptides are obtained with molecular weight ranges of greater than about 50,000Da, about 35,000Da to about 50,000Da, about 25,000Da to about 35,000Da, and about 10,000Da to about 30,000Da.

In a further embodiment, the present invention comprises separating the one or more recovered polypeptides to recover individual polypeptides. Separation can be performed according to any well known techniques such as for example chromatography, one dimensional gel electrophoresis, two dimensional electrophoresis or the like.

The inventors have further analysed and identified the individual polypeptides separated by the method of the present invention.

Polypeptides recovered by the method of the invention are connective tissue derived polypeptides. The inventors have found that the recovered polypeptides and mixtures thereof have enhanced and/or different pharmacological activities to the GAG-peptide/polypeptide mixtures.

Accordingly, in a second aspect the present invention provides a connective tissue derived polypeptide, obtainable by the method of the invention, having anti-arthritic or anti-inflammatory activity. In one embodiment, polypeptides of the invention and mixtures thereof reduce rear paw inflammation in rats with collagen induced arthritis. In another embodiment, polypeptides of the invention and mixtures thereof decrease tail inflammation in rats with collagen induced arthritis. In another embodiment, polypeptides decrease fore paw inflammation in rats with collagen induced arthritis. In yet another embodiment, polypeptides of the invention and mixtures thereof decrease weight loss in rats with collagen induced arthritis.

As used herein the term "derived" shall be taken to indicate that a specified integer may be obtained from a source, albeit not necessarily directly from that source.

In an alternate embodiment, the polypeptides of the present invention are connective tissue derived polypeptides obtainable by the methods of the present invention having a molecular weight in the range of about 10,000Da and about 50,000Da and having anti-arthritic or anti-inflammatory.

In an alternate embodiment, the polypeptides of the present invention are connective tissue derived polypeptides obtainable by the methods of the present invention having a molecular weight in the range of about 15,000Da and about 25,000Da and having anti-arthritic or anti-inflammatory.

In an alternate embodiment, the polypeptides of the present invention are connective tissue derived polypeptides obtainable by the methods of the present invention having a molecular weight in the range of about 25,000Da and about 50,000Da and having anti-arthritic or anti-inflammatory.

In another embodiment, the polypeptides of the present invention are connective tissue derived polypeptides obtainable by the methods of the present invention, having a molecular weight in the range of about 50,000Da and about 100,000Da and having anti-arthritic or anti-inflammatory.

Mixtures of polypeptides which have a molecular weight in a desired range are clearly contemplated.

In another embodiment, individual polypeptides obtainable by the methods of the invention having anti-arthritic or anti-inflammatory are contemplated.

In a preferred embodiment, the present invention provides a connective tissue derived polypeptide having anti-arthritic or anti-inflammatory selected from the group consisting of:

(a) a mixture of connective tissue derived polypeptides having a combined molecular weight of about 45,000Da to about 55,000Da, more preferably 48,000Da to about 52,000Da, more preferably about 50,000Da as determined by SDS/PAGE

(b) a connective tissue derived polypeptide having a molecular weight of about 35,000Da to about 45,000Da, more preferably 35,000Da to about 40,000Da, more preferably about 38,000Da as determined by SDS/PAGE

(c) a connective tissue derived polypeptide having molecular weight of about 20,000Da to about 25,000Da, more preferably 20,000Da to about 23,000Da, more preferably about 22,000Da as determined by SDS/PAGE

(d) a connective tissue derived polypeptide having molecular weight of about 15,000Da to about 25,000Da, more preferably 18,000Da to about 22,000Da, more preferably about 20,000Da as determined by SDS/PAGE

(e) a connective tissue derived polypeptide having molecular weight of about 14,000Da to about 22,000Da, more preferably 16,000Da to about 20,000Da, more preferably about 18,000Da as determined by SDS/PAGE

(f) a connective tissue derived polypeptide having molecular weight of about 15,000Da to about 25,000Da, more preferably 17,000Da to about 21,000Da, more preferably about 19,000Da as determined by SDS/PAGE

(g) a connective tissue derived polypeptide having molecular weight of about 15,000Da to about 25,000Da, more preferably 18,000Da to about 23,000Da, more preferably about 20,000Da as determined by SDS/PAGE

(h) a connective tissue derived polypeptide having molecular weight of about 15,000Da to about 23,000Da, more preferably 17,000Da to about 20,000Da, more preferably about 19,000Da as determined by SDS/PAGE

(i) a connective tissue derived polypeptide having molecular weight of about 20,000Da to about 25,000Da, more preferably 20,000Da to about 23,000Da, more preferably about 22,000Da as determined by SDS/PAGE

(j) a connective tissue derived polypeptide having molecular weight of about 18,000Da to about 24,000Da, more preferably 19,000Da to about 23,000Da, more preferably about 21,000Da as determined by SDS/PAGE

Preferably, the connective tissue derived mixture of polypeptides in paragraph (a) *supra* have an isoelectric point (pI) of about 3.5 to about 4.5, and more preferably a pI value of about 3.9 as determined by isoelectric focussing.

Preferably, the connective tissue derived polypeptide in paragraph (b) *supra* has an isoelectric point (pI) of about 6 to about 6.5, and more preferably a pI value of about 6.3 as determined by isoelectric focussing.

Preferably, the connective tissue derived polypeptide in paragraph (c) *supra* has an isoelectric point (pI) of about 6.5 to about 7, and more preferably a pI value of about 6.8 as determined by isoelectric focussing.

Preferably, the connective tissue derived polypeptide in paragraph (d) *supra* has an isoelectric point (pI) of about 7.5 to about 8.5, and more preferably a pI value of about 7.8 as determined by isoelectric focussing.

Preferably, the connective tissue derived polypeptide in paragraph (e) *supra* has an isoelectric point (pI) of about 8 to about 8.5, and more preferably a pI value of about 8.2 as determined by isoelectric focussing.

Preferably, the connective tissue derived polypeptide in paragraph (f) *supra* has an isoelectric point (pI) of about 8 to about 8.5, and more preferably a pI value of about 8.3 as determined by isoelectric focussing.

Preferably, the connective tissue derived polypeptide in paragraph (g) *supra* has an isoelectric point (pI) of about 8.3 to about 8.8, and more preferably a pI value of about 8.6 as determined by isoelectric focussing.

Preferably, the connective tissue derived polypeptide in paragraph (h) *supra* has an isoelectric point (pI) of about 8.8 to about 9.5, and more preferably a pI value of about 9.1 as determined by isoelectric focussing.

Preferably, the connective tissue derived polypeptide in paragraph (i) *supra* has an isoelectric point (pI) of about 6 to about 6.5, and more preferably a pI value of about 6.2 as determined by isoelectric focussing.

Preferably, the connective tissue derived polypeptide in paragraph (j) *supra* has an isoelectric point (pI) of about 6.8 to about 7.5, and more preferably a pI value of about 7.2 as determined by isoelectric focussing.

Individual polypeptides of the invention have been identified as fragments of known proteins. Peptides of up to about 30 amino acids were produced by subjecting a polypeptide to trypsin digestion and then to Matrix Assisted Laser Desorption Ionisation (MALDI) mass spectrometry. The peptides have been compared to known proteins provided in reference databases.

Accordingly, in another embodiment, the present invention provides an isolated polypeptide or polypeptide fragment obtainable by the method of the invention comprising the sequences and % match with known proteins as disclosed in Figure 4, wherein the polypeptide or polypeptide fragment has anti-arthritic or anti-inflammatory.

Preferably, the connective tissue derived polypeptide in paragraph (b) *supra* comprises one or more of the following sequences following trypsin digestion:

(K)LGNNV DFR(I)

(R)IESLP IKPR(G)

(R)HLYPN GLPEE YSFLT TFR(M)

(K)IMIGV ER(S)

(R)SSATL FVDCN R(I)

Preferably, the connective tissue derived polypeptide in paragraph (c) *supra* comprises one or more of the following sequences following trypsin digestion:

(K)SVSFS YK(G)

(K)IMIGV ER(S)

(K)LGNNV DFR(I)

(R)IESLP IKPR(G)

(K)HWSIW QIQDS SGK(E)

(R)IGQDD LPGFD LISQF QIDK(A)

(R)HLYPN GLPEE YSFLT TFR(M)

(K)GLDGS LQTAA FSNLP SLFDS QWHK(I)

(K)IMIGV ER(S)

(R)SSATL FVDCN R(I)

Preferably, the connective tissue derived polypeptide in paragraph (d) *supra* comprises one or more of the following sequences following trypsin digestion:

(K)SVSFS YK(G)

(K)IMIGV ER(S)

(K)LGNNV DFR(I)

(R)IESLP IKPR(G)

(K)HWSIW QIQDS SGK(E)

(R)IGQDD LPGFD LISQF QIDK(A)

(R)HLYPN GLPEE YSFLT TFR(M)

(K)GLDGS LQTAA FSNLP SLFDS QWHK(I)

(K)IMIGV ER(S)

(R)SSATL FVDCN R(I)

Preferably, the connective tissue derived polypeptide in paragraph (e) *supra* comprises one or more of the following sequences following trypsin digestion:

(K)SVSFS YK(G)

(K)IMIGV ER(S)

(K)LGNNV DFR(I)

(R)IESLP IKPR(G)

(K)HWSIW QIQDS SGK(E)

(R)HLYPN GLPEE YSFLT TFR(M)

(K)GLDGS LQTAA FSNLP SLFDS QWHK(I)

(R)SSATL FVDCN R(I)

Preferably, the connective tissue derived polypeptide in paragraph (f) *supra* comprises one or more of the following sequences following trypsin digestion:

(K)LGNNVDFR(I)

(R)IESLPIKPR(G)

(R)IGQDD LPGFD LISQF QIDK(A)

(R)HLYPN GLPEE YSFLT TFR(M)

(R)SSATL FVDCN R(I)

Preferably, the connective tissue derived polypeptide in paragraph (g) *supra* comprises one or more of the following sequences following trypsin digestion:

(K)SVSFS YK(G)

(K)IMIGV ER(S)

(K)LGNNV DFR(I)

(R)IESLP IKPR(G)

(K)HWSIW QIQDS SGK(E)

(R)IGQDD LPGFD LISQF QIDK(A)

(R)HLYPN GLPEE YSFLT TFR(M)

(K)IMIGV ER(S)

(R)SSATL FVDCN R(I)

Preferably, the connective tissue derived polypeptide in paragraph (h) *supra* comprises one or more of the following sequences following trypsin digestion:

(K)LGNNV DFR(I)

(R)IESLP IKPR(G)

(R)IGQDD LPGFD LISQF QIDK(A)

(R)HLYPN GLPEE YSFLT TFR(M)

(R)SSATL FVDCN R(I)

Preferably, the connective tissue derived polypeptide in paragraph (i) *supra* comprises one or more of the following sequences following trypsin digestion:
(K)SVSFS YK(G)
(K)IMIGV ER(S)
(K)LGNNV DFR(I)
(R)IESLP IKPR(G)
(K)HWSIW QIQDS SGK(E)
(R)IGQDD LPGFD LISQF QIDK(A)
(R)HLYPN GLPEE YSFLT TFR(M)
(K)IMIGV ER(S)
(R)SSATL FVDCN R(I)

Preferably, the connective tissue derived polypeptide in paragraph (j) *supra* comprises one or more of the following sequences following trypsin digestion:
(K)SVSFS YK(G)
(K)IMIGV ER(S)
(K)LGNNV DFR(I)
(R)IESLP IKPR(G)
(K)HWSIW QIQDS SGK(E)
(R)IGQDD LPGFD LISQF QIDK(A)
(R)HLYPN GLPEE YSFLT TFR(M)
(K)IMIGV ER(S)
(R)SSATL FVDCN R(I).

Preferably, the connective tissue derived polypeptide in paragraph (a) *supra* comprises an amino acid sequence having substantial identity to the sequence of COMP protein as depicted in Figure 5 or homologue or derivative thereof.

Preferably, the connective tissue derived polypeptide in paragraphs (b)-(j) *supra* comprises an amino acid sequence having substantial identity to the sequence of Type IX collagen alpha 1 chain as depicted in Figure 6 or homologue or derivative thereof.

Proteins and derivatives

The terms "protein", "polypeptide", or "peptide", when used herein refer to amino acids in a polymeric form of any length. Said terms also include known amino acid modifications such as disulfide bond formation, cysteinylation, oxidation, glutathionylation, methylation, acetylation, farnesylation, biotinylation, stearoylation, formylation, lipoic acid addition, phosphorylation, sulfation, ubiquitination, myristoylation, palmitoylation, geranylgeranylation, cyclisation, oxidation,

deamidation, dehydration, glycosilation, acylation, and radio labels as well as non-naturally occurring amino acid residues, L-amino residues and D-amino acid residues. As used herein the term "substantial identity" means that the detected protein comprises an amino acid sequence having at least about 80% identity to the reference protein, more preferably at least about 85% identity, even more preferably at least about 90% identity and still even more preferably at least about 95% identity or at least about 97% identity or at least about 99% identity to the referenced protein.

"Homologues" or "Homologs" of a protein of the invention are those peptides, oligopeptides, polypeptides, proteins and enzymes which contain amino acid substitutions, deletions and/or additions relative to the said protein with respect to which they are a homologue, without altering one or more of its functional properties, in particular without reducing the activity of the resulting homologue of said protein. For example, a homologue of said protein will consist of a bioactive amino acid sequence variant of said protein. To produce such homologues, amino acids present in the said protein can be replaced by other amino acids having similar properties, for example hydrophobicity, hydrophilicity, hydrophobic moment, antigenicity, propensity to form or break α-helical structures or β-sheet structures, and so on.

"Derivatives" of a polypeptide of the invention are those peptides, oligopeptides, polypeptides, proteins and enzymes which comprise at least about 5 contiguous amino acid residues of said polypeptide but which retains the biological or enzymatical activity of the polypeptide where it derived from; and which may further comprise additional naturally-occurring, altered glycosolylated, acylated, or non-naturally occurring amino acid residues compared to the amino acid sequence of a naturally-occurring form of said polypeptide. Alternatively, or in addition, a derivative may comprise one or more amino acids substituents compared to the amino acid sequence of a naturally-occurring form of said polypeptide, for example a recorder molecule or other ligand covalently or non-covalently bound to the amino acid sequence such as, for example, a recorder molecule which is bound thereto to facilitate its detection. A derivative of a protein retains the biological or enzymatic activity of the protein where it derived from.

Treatment, protection and restoration of connective tissues and connective tissue disease

The effects observed for the polypeptides on inflammation and collagen induced arthritis in rats also provide application for the polypeptides, and mixtures thereof, in the treatment, protection and restoration of connective tissues, particularly in

inflammatory and degenerative tissue diseases such as rheumatoid arthritis and osteoarthritis.

Polypeptides according to the invention that have been shown to have anti-inflammatory and/or anti-arthritic activity can be further tested for safety and efficacy in animal models, such as for example in small rodent models, and then proceed to clinical trials in humans, if desired. Naturally, for veterinary applications, no clinical trial in humans is required. Those polypeptides that are safe and efficacious in animals or humans can be administered to an appropriate subject to treat a connective tissue disease, or alternatively to protect against connective tissue disease by the process of tolerisation.

Rodent models of connective tissue disease are well known. For example, rat models for collagen induced arthritis and pristane induced arthritis rats are described in Lu *et al* (1999) (*supra*). Murine collagen induced models of arthritis are also described in Myers *et al* (2002) (*supra*).

Methods and pharmaceutical formulations for the treatment of autoimmune arthritis and animal models therefore in mammals, including humans, by the oral, enteral or by-inhalation administration of whole collagen type II protein or biologically active peptide fragments of collagen type II are described in US 5,399347 (Trentham *et al*). Further, methods and formulations for evaluating the efficacy of oral type II collagen in the treatment of juvenile rheumatoid arthritis (JRA) are described in Barnett et al (1996) (*supra*).

Intranasal formulations are described and administration of larthrytic collagen type II and larthrytic collagen type IX are described in Lu *et al* (1999) (*supra*).

A third aspect of the invention provides for the use of a connective tissue derived polypeptide or derivative or fragment thereof having anti-arthritic or anti-inflammatory, in the preparation of a medicament for the treatment of connective tissue disease in a subject, wherein said polypeptide is obtainable by the methods of the first aspect of the present invention.

In a related embodiment the invention provides for the use of a connective tissue derived polypeptide or derivative or fragment thereof having anti-arthritic or anti-inflammatory, in the preparation of a medicament for the treatment of connective tissue disease in a subject, wherein said polypeptide is a polypeptide according to the second aspect of the invention.

"Treatment" includes both prophylactic and therapeutic measures to prevent the onset and appearance of a connective tissue disease as well as to prevent the onset and appearance of an abnormal immune response against the body's own tissues involved in

autoimmune disease. The term also encompasses the suppression or mitigation of the abnormal (cell and/or humoral) immune response to the body's own collagen or more generally cartilage as well as the alleviation or elimination of clinical symptoms after the onset (ie. clinical manifestation) of autoimmune disease.

"Autoimmune disease" is defined as a malfunction of the immune system of mammals, in which the immune system fails to distinguish between foreign substances within the mammal and/or autologous tissues or substances and, as a result, treats autologous tissues and substances as if they were foreign and mounts an immune response against them.

In related aspects, the present invention also provides pharmaceutical compositions for the treatment and repair of connective tissue in mammals comprising a therapeutically effective amount of the polypeptide of the present invention or derivative or fragment thereof having anti-arthritic or anti-inflammatory activity, (such as vaccine and/or adjuvant compositions as discussed herein) and a pharmaceutically acceptable carrier, diluent or excipients (including combinations thereof).

In one embodiment, the pharmaceutical composition may comprise two components, wherein a first component comprises a polypeptide and a second component which comprises adjuvant thereof. The first and second component may be delivered sequentially, simultaneously or together, and even by different administration routes.

The pharmaceutical compositions may be for human or animal usage in human and veterinary medicine and will typically comprise any one or more of a pharmaceutically acceptable diluent, carrier or excipient. Acceptable carriers or diluents for therapeutic use are well known in the pharmaceutical art, and are described for example in *Remington's Pharmaceutical Sciences* Mack Publishing Co. (A.R. Gennaro edit. 1985). The choice of pharmaceutical carrier , excipient or diluent can be selected with regard to the intended route of administration and standard pharmaceutical practice. The pharmaceutical compositions may comprise as, or in addition to, the carrier, excipient or diluent, any suitable binder, lubricant, suspending agent, coating agent, or solubilizing agent.

Preservatives, stabilisers, dyes and flavouring agents may be provided in the pharmaceutical composition. Examples of preservatives include sodium benzoate, sorbic acid and esters or p-hydroxybenzoic acid. Anti-oxidants and suspending agents may also be used.

There may be different composition/ formulation requirements dependant on the different delivery systems. By way of example, the pharmaceutical composition of the present invention may be formulated to be delivered using a mini-pump or by a mucosal route, for example, as a nasal spray or aerosol for inhalation or ingestible solution, or parenterally in which the composition is formulated by an injectable form, for delivery by, for example, an intravenous, intramuscular or subcutaneous route. Alternatively, the formulation may be designed to be delivered by both routes. Preferably, the formulation is administrable in a non-invasive form. Preferably, the pharmaceutical composition is formulated to be delivered orally or nasally.

Where the agent is to be delivered mucosally through the gastro-intestinal mucosa, it should be able to remain stable during transit through the gastro-intestinal tract; for example, it should be resistant to proteolytic degradation, stable antacid, pH and resistant to the detergent effects of bile.

Where appropriate, the pharmaceutical compositions can be administered by inhalation, orally or intranasally, in the form of suppository or pessary, topically in the form of a lotion, solution, cream, ointment, or dusting powder, by use of a skin patch, orally in the form of tablets containing excipients such as starch or lactose, or in capsules or ovules either alone or in admixture with excipients, or in the form of elixirs, solutions or suspensions containing flavouring or colouring agents, or they can be injected parenterally for example, intravenously, intramuscularly or subcutaneously. For parenteral administration, the compositions may be best used in the form of a sterile aqueous solution which may contain other substances, for example enough salts or monosaccharides to make the solution isotonic with blood. For buccal or sublingual administrations, the compositions may be administered in the form of tablets or lozenges which can be formulated in a conventional manner.

The foregoing embodiments apply *mutatis mutandis* to prophylactic or therapeutic vaccine compositions.

Preferably, the compositions of the invention are administered by a non-invasive route. Preferably the non-invasive route comprises oral administration, or enteral administration, nasal administration or by inhalation.

Accordingly, in a fourth aspect the invention provides a prophylactic or therapeutic vaccine composition for eliciting an immune response against connective tissue derived polypeptides, said composition comprising a connective tissue derived polypeptide or derivative or fragment thereof in combination with a pharmaceutically acceptable carrier, excipient, diluent and/or adjuvant.

A "prophylactic" vaccine is a vaccine which is administered to naive individuals to prevent disease development, such as by stimulating active immunity.

A "therapeutic" vaccine is a vaccine which is administered to individuals with an existing connective tissue disease to reduce or minimise the disease or to abrogate the immunopathological consequences of the connective tissue disease.

Preferably, each polypeptide or derivative or fragment thereof having anti-arthritic or anti-inflammatory activity, is formulated with a suitable adjuvant to protect a subject from developing a connective tissue disease.

In a fifth aspect the invention provides a method of treating or preventing a connective tissue disease in a subject comprising administering to the subject an effective amount of a pharmaceutically acceptable composition comprising a connective tissue derived polypeptide having anti-arthritic or anti-inflammatory activity wherein said polypeptide is obtainable by the methods of the first aspect of the invention.

In a related aspect the invention provides a method of treating or preventing a connective tissue disease in a subject comprising administering to the subject an amount of a pharmaceutically acceptable composition comprising a connective tissue derived polypeptide having anti-arthritic or anti-inflammatory activity, wherein said polypeptide is a polypeptide according to the second aspect of the invention.

Preferably, administration of the polypeptides is an effective means of suppressing a T-cell mediated or T-cell dependant connective tissue disease, more particularly an autoimmune disease, and more particularly arthritis.

Preferably, administration is by a non-invasive route. More preferably, the polypeptides or derivatives or fragments thereof are administered by oral, enteral, nasal or by-inhalation routes.

Oral, enteral, nasal or by-inhalation administration of fragments of polypeptides according to the present invention is expected to be more convenient and/or specific in eliciting autoimmune suppression than administration of the entire polypeptide sequence without risk of sensitising the mammal to the autoimmune response inducing portions of the connective tissue protein(s).

Additional immunosuppressive polypeptide fragments or peptides can be identified by routine experimentation in light of the present specification, claims and figures. A method for identifying peptide fragments having stimulatory activity is described, for example, in US5,399342.

In a sixth embodiment the invention provides a method of inducing cartilage formation in an individual, comprising administering to the individual an effective

amount of a connective tissue derived polypeptide having anti-inflammatory or anti-arthritic activity wherein said polypeptide is obtainable by the methods of the first aspect of the invention. In a related embodiment the polypeptide is a polypeptide according to the second aspect of the invention.

Modes for Carrying Out the Invention

Experimental Protocols

Bovine, ovine, cervine or porcine tracheal cartilage or nasal cartilage, chicken sternal cartilage, or skeletal shark cartilage or deer antler cartilage were freed of adhering soft tissues mechanically or as described previously US Patent 5,399.347 Mar 1995, US Patent 5,364,845 Dec 1996, US Patent 6,025,327 Feb 2000). These cleaned hyaline cartilages were rinsed with water, minced into 1 mm or 3 mm sizes, freeze dried and stored at –20 °C. Bovine tracheal chondroitin sulfate A (ChSA) was purchased from Sigma Chemical Co, USA or was obtained as a gift from Bioiberica, Barcelona, Spain (batch 1/0015, batch 05/2001, batch 18/11/99). All other chemicals were of analytical grade and were purchased from local suppliers.

Release of Glycosaminoglycan Peptide (GAG-peptide) Complexes and polypeptides from the Cartilage Powders

Studies on the kinetics of release of the GAG-peptides and polypeptides from the cartilage powders were performed using different buffers (eg sodium or calcium acetate or dilute acetic acid) to give various products referred to herein as "Peptacan(s)" and was undertaken under a variety of conditions. The objective of these experiments was to determine the effects of (i) particle size - 3mm,5mm, (ii) different pHs eg. pH range 3.5-7.0, (iii) different temperatures, 4°C, 25°C and 37°C, and (iv) animal species and tissue locations on the rate of autolysis and product release into the aqueous phase. All the experiments were performed, with stirring and release of sulphated GAGs and polypeptides monitored over 24 hours. In the initial studies undertaken, the primary observation was that subjecting particles of cartilages to autolysis in aqueous buffers maintained within the pH range of 4.0-7.0, particularly 4.5, preferably at 37°C for periods up to 24 hours specifically released more than 80% of the total sulfated glycosaminoglycans (S-GAG) into solution. Studies also showed that the rate of release was dependent on the cartilage particle size, the smaller preparations undergoing more rapid release. However, by 24 hours the yields obtained were the same. The pH and temperature were found to be important determinants of the rate of release which indicated that the release process was mediated by endogenous enzymes

present within the solid tissues. This proposed mechanism was confirmed by undertaking autolysis experiments in the absence and presence of specific enzyme inhibitors. Since it was found that the addition of N-ethylmaleimide produced the most significant inhibition of GAG-peptide and polypeptides release into the aqueous medium we consider that the cysteine class of proteinases, such as the Cathepsins, were the major, but not exclusive, contributors to the autolytic process.

The aqueous phase was separated from the cartilage powders by filtration and the filtrate centrifuged to remove fine particles and then neutralised to pH 7.0 by addition of an alkaline solution containing the desired cation. These Peptacan solutions after chemical analysis were either freeze dried and used directly for pharmacological studies. The freeze dried Peptacans were used as stock material for the preparation of dialysed and fractionated preparations as described below.

Alternatively the Peptacans could be isolated from the aqueous solutions obtained from the cartilage digests by precipitation with excess quantities of acetone, ethanol or methanol, usually by adding 3-5X the volume of the aqueous extracts. The precipitates so obtained would be washed with absolute ethanol and dried under vacuum then stored in a vacuum desiccator.

The process of the present invention is essentially non-disruptive leaving the type II collagen matrix and cells of the tissue intact, and the absence of DNA in the autolysis media. The efficiency of the autolytic process was also influenced by the animal species and anatomical location from where the cartilage was derived as well as the nature of the buffers used.

For the further experiments described herein, the results were obtained following subjecting bovine tracheal cartilage to autolysis with calcium acetate buffer. For the purpose of convenience, the product obtained by this process is referred to herein as Calcium Peptacan (CaP).

Separation of Glycosaminoglycan Peptides (GAG-peptides) from polypeptides in Peptacan preparations by Ion-exchange solid phase media

Freeze-dried Peptacans was dissolved in 0.1M calcium chloride buffered with Tris-HCl to a pH of 7.2 (application buffer) to afford sample concentrations of 4.0 mg/ml. To these solutions was added pre-swollen DEAE-Sepharose-6B to achieve a final concentration of the ion exchanger of 100mg/mL. This mixture was maintained at room temperature with gentle agitation for 16 hours in 5mL stoppered centrifuge tubes. The tubes were then centrifuged at 1000rpm for 5 mins and the supernatant decanted

off. To the remaining pellet was added 1mL of the application buffer and the tubes gently shaken, then centrifuged again.

Filtration

The supernatant and washings were added to the original supernatant which was subjected to filtration using a 500Da cut-off cellulose acetate ultrafiltration membrane (YC05, Millipore Australia Pty Ltd, Sydney, Australia). The filtrated polypeptide solution (de-salted) was then freeze-dried and stored at –20°C.

Further separation /filtration of the polypeptide solution can be achieved using any one or combination of techniques such as, for example, gel filtration, ultrafiltration, SDS PAGE electrophoresis, 2D gel electrophoresis, spiral cartridge separation and reverse phase HPLC using established methods (see for example,Eyre D, et al. Collagen type IX: evidence for covalent linkages to type II collagen in cartilage. FEB 220:337-341,1987).

Further separation of the polypeptide solution can be performed to obtain polypeptides having a molecular weight of greater than 10,000Da.

BIOLOGICAL ACTIVITY

Determination of collagen or collagen peptide content in preparations by assay for hydroxyproline

The collagen content of polypeptides separated by ion exchange was estimated by measuring the concentration of the amino acid hydroxyproline which is unique to this protein. Each freeze dried sample was directly dissolved in H_2O (10 mg/ml) and then hydrolysed in 5 N HCl at 110 °C for 24 h. The hydrolysed sample solution was neutralised to pH 7 before dilution and analysis. The hydroxyproline concentration in these solutions was determined using the method of Stegman and Stalder (Stegman H and Stalder K. Determination of Hydroxyproline. Clin. Chim. Acta 18:267-273, 1967) by using a L-hydroxyproline standard and measuring the absorbance at 562 nm after the addition of chloramine T and p-dimethylaminobenzaldehyde to develop the chromophore. The hydroxyproline concentration was multiplied by 7.4 to give an estimate of the collagen content.

Determination of Protein content of preparations content by the Bicinchoninic Acid (BCA) assay

The total protein content of polypeptide samples was determined using BCA assay (Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD,

Fujimoto EK, Goeke NM, Olson BJ and Klenk DC. Anal. Biochem. 150, 76–85, 1985). Freeze dried polypeptide samples were directly dissolved in H_2O to provide a 2.0 mg/ml solution and 20 µl of each sample solution was added to a well of 96-well plates. Just prior to assay, 50 parts of reagent 1 (0.4% NaOH; 1.7% Na_2CO_3; 0.95% $NaHCO_3$; 1.0% bicinchoninic acid; 0.16% Na_2-tartrate) was mixed with reagent 2 (4% $CuSO_4.5H_2O$). Two hundred micro litres of this working reagent was added to the sample solution. After incubation at 37 °C for 60 min the absorbance A_{562} was read using a Thermomax microplate reader. Bovine serum albumin (BSA) or highly purified gelatine (Gibco) at 0–10 µg/well were used to construct a standard curve.

Preparation of INR-195

In this procedure the supernatant and washings containing the polypeptide components obtained from ion exchange separation of calcium peptacan using DEAE-Sepharose were transfered to a reservior connected via a peristaltic pump to a 10kD PLAC (PL series Cellulose or polysulfone 0.93 square metre spiral cartridge (Millipore Australia Pty Ltd). The peptide containing solution is then subjected to tangental flow filtration for 6 hours by addition of purified water to the reservior to dialysis off the small inorganic ions used for ion exchange procedure as well as peptides with molecular weights below the cut-off of the membrane. The retentiate was then concentrated by diafiltration in the same apparatus, collected and water removed by freeze drying. In such a procedure the product obtained by this method was designated as INR-195 and was used for the rat CIA experiments described herein.

Rat collagen induced arthritis (CIA)

Female Wistar rats (160-180 gm) were inoculated with 250 µg bovine tracheal Collagen-II, given as 6 divided injections into the tailbase on Day O.

INR-195, dissolved in water and was administered orally either:

(i) once daily for 15 days = prophylactic protocol or

(ii) beginning Day 15 or Day 17 = therapeutic protocol.

The following signs of arthritis were assessed from Day 11 onwards:

Rear paw swelling, tail swelling, fore paw inflammation (scored on a scale 0-4+) and an overall arthritis score (also scored 0-4+) assigned on the basis of overall inflammation and other signs of disease e.g. piloerection, diminished mobility, poor grooming etc.

Gastric susceptibility to a single dose of 50 mg/kg ibuprofen was assessed after fasting overnight, administering ibuprofen in 10 ml/kg water and terminating animals

2.5 hours later. Haemorrhagic lesions were enumerated and scored for severity of a scale 0-4.

The results are shown in Tables 2, 3 and 4.

Analysis of polypeptides separated by ion exchange using SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Freeze dried polypeptide samples were dissolved in H_2O and then mixed 1:1 with 2x sample loading buffer (0.07 M TrisHCl, 1.5% SDS, 20% glycerol, 0.2M DTT and 0.1% BPB) to achieve the final concentrations of 4.0 – 20 mg/ml. The samples were boiled in a water bath for 5 min. 20 µl of above samples were loaded into the wells of 8 – 16 % pre-cast Tris-glycine gel (Norvex). SeeBlue pre-stained low molecular weight range protein markers (Norvex) were loaded into wells on the left-hand side of the gel and electrophoresis was performed at 125 V for 2 h. The gel was stained in Coomassie blue R250 solution (40% ethanol, 10% acetic acid and 0.2% Coomassie R250) for 30 min and destained in a solution containing 10% ethanol and 7.5% acetic acid for 16 h. The gel was dried in a Bio-Rad Gelair drier.

SDS-PAGE of the polypeptides separated from the GAG-peptides by the ion exchange procedure showed the presence of a number of bands the most abundant of which had molecular weights of 36kDaltons or less (Figure 2).

Analysis of polypeptides separated by ion exchange using two-Dimensional gradient SDS-PAGE

Freeze dried polypeptide samples (6.5mg) were dissolved in H_2O and sonicated. Samples were then centrifuged at 20,000g for 10 minutes then were loaded via in-gel rehydration methods for Isoelectric Focusing (IEF) using 11 cm gradient strips over a pH of 3-10. First Dimension focusing was at 35,000Vh. The second dimension used separating gradient 8-18%T criterion format polyacrylamide slab gels. This second dimension electrophoresis was run at 1 hour @ 5mA/gel and 4 hours @ 15mA/gel. Gels were stained using SYPRO Ruby fluorescent stain then scanned to produce a digital image.

Two dimensional electrophoresis of the polypeptides separated from the GAG-peptides by the ion exchange procedure revealed the presence of at least 21 polypeptides (Figure 3).

Tryptic digestion and Matrix assisted laser desorption ionisation (MALDI) mass spectrometry

Following two-dimensional gradient SDS-PAGE of samples they were subjected to an in gel 16 hour tryptic digest at 37°C. The resulting peptides were extracted from the gel with a 10% (v/v) acetonitrile, 1% (v/v) TFA solution. The samples were then cleaned up and concentrated using ZipTip. A 1μL aliquot of each was spotted onto a sample plate with 1μL of matrix (a-cyano-4-hydroxycinnamic acid, 8mg/mL in 70% v/v AcN, 1% v/v TFA) and allowed to air dry. Matrix assisted laser desorption ionisation (MALDI) mass spectrometry was then performed with a Micromass Maldi Time of Flight Mass Spectrometer. A nitrogen laser (337 nm) was used to irradiate the sample. The spectra were acquired in reflectron mode in the mass range 750 to 3500 Da. A near point calibration was applied.

The peptide masses of the monoisotopic peak of the peptides from this analysis were searched against Bovine using ProteinLynx on MassLynx and Mammalia data bases using PeptIdent on Expasy. Positive identification of 17 of the polypeptides took account of the percentage of sequence coverage, how well the masses matched the significant peaks in the MS spectra, the number of missed cleavages (if missed cleavages were present their location in the sequence was critical) and how well the MW and pI of the identified protein match. These sequences and their % match with known proteins are shown in Figure 4.

The most abundant polypeptide fragments present in those isolated from CaP by the ion exchange method were derived from the Type IX collagen alpha 1 chain (Molecular weight: 20907). Good matches of the trypsin generated products were found for: Polypeptide 10, Polypeptide 13, Polypeptide 14, Polypeptide 15, Polypeptide 16, Polypeptide 17, Polypeptide 18, Polypeptide 20 and Polypeptide 21 (Figure 4). The presence of these Type IX collagen alpha 1 chain was consistent with the proteolytic action of the endogenous proteases known to be present in cartilage, such as the matrix metallo proteases and the cathepsins

The 4 non-collagenous domains (including NC1-4) along the Type IX collagen alpha 1 would be expected to be the most susceptible to proteolytic cleavage by these enzymes. This view was supported by the finding of a fragment (Polypeptide 2) of Bovine Cartilage oligomeric matrix protein (COMP)(Mw: undefined) in the polypeptides of the CaP preparation. COMP through its C-terminal domain interacts with the non-collagenous domains (NC1-4) of the Type IX collagen alpha 1 chain Together these two proteins play key structural roles in the assembly of the extracellular matrix of cartilage and, as already mentioned, the Type IX collagen is

located on the surface of the type II collagen fibrils where it serves as cross linking units between fibrils and itself. It was therefore surprising to find that the polypeptides present in the CaP preparation did not contain any peptides originating from the Type II collagen molecule. However, it is possible that such polypeptide fragments, if produced by the autolytic digestive process, would be less soluble in aqueous solution at neutral pH and were therefore precipitated and removed during the filtration step used prior to ion exchange treatment.

Importantly, the present findings highlights the susceptibility of the Type IX collagen alpha 1 chain to the hydrolytic activities of the endogenous proteanases within cartilage. In addition, as this minor collagen provides such an important role in maintaining the structural integrity of cartilage, its cleavage, even if the associated type II collagen fibrils are not damaged, would lead to impairment in the mechanical functions of this tissue. Since it is known that Type II collagen content of articular cartilage remains unperturbed in the very early stages of OA it is highly likely that it is the proteolytic modification of Type IX collagen which represents the initial "pathological lesion" in this disease.

Fragments of Bovine Serum Albumin (Molecular weight: 69294) (Polypeptide 7, Polypeptide 8, Polypeptide 9, Polypeptide 11, Polypeptide 12) were also found in the polypeptide fraction of CaP (Figure 4). The presence of fragments of this protein was not unexpected in view of the large reservoir available in the blood of tissues adjacent to the tracheal cartilage.

Neutrophil cytosol factor 1 (Molecular weight: 45346) (Polypeptide 1 and Polypeptide 9), Odorant-binding protein (Molecular weight: 18503) (Polypeptide 19) have not previously been reported to be present in cartilage and their functions, if any, are therefore unknown. Odorant-binding protein has however, been identified in bovine nasal mucosa which is physically close to the trachea. It is possible that this protein is sequestered to the tracheal cartilage and is not in fact a chondrocyte biosynthetic product. Again this question still remains to be answered.

Dated this Seventeenth day of June 2003

Chondrotec Pty Limited
Patent Attorneys for the Applicant:

F B RICE & CO

Figure 1

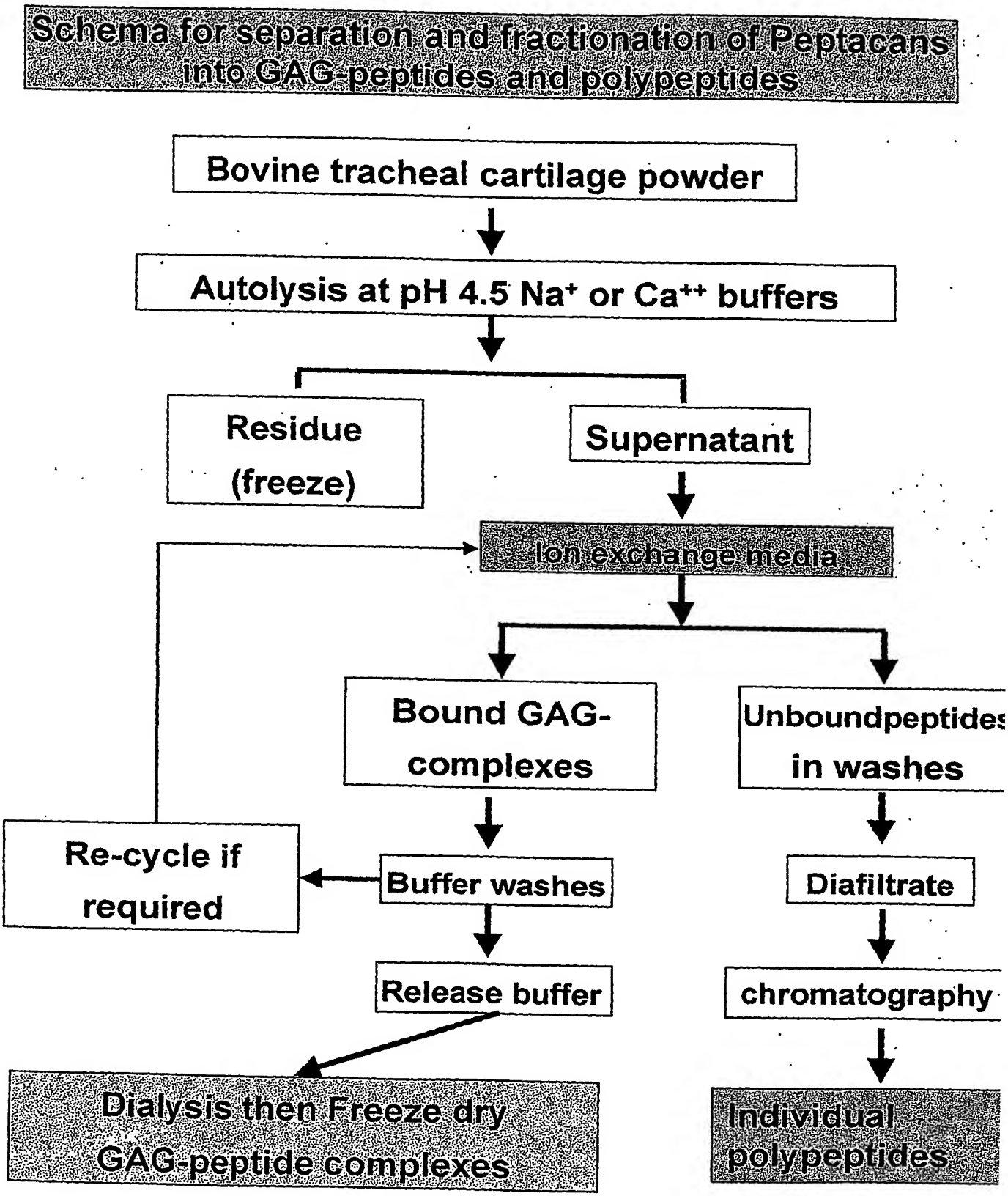

Schema for separation and fractionation of Peptacans into GAG-peptides and polypeptides
Bovine tracheal cartilage powder
Autolysis at pH 4.5 Na+ or Ca++ buffers
Residue (freeze)
Supernatant
Ion exchange media
Bound GAG-complexes
Unboundpeptides in washes
Re-cycle if required
Buffer washes
Diafiltrate
Release buffer
chromatography
Dialysis then Freeze dry GAG-peptide complexes
Individual polypeptides

Figure 2

SDS-PAGE of Proteins (polypeptides) Isolated from CaP

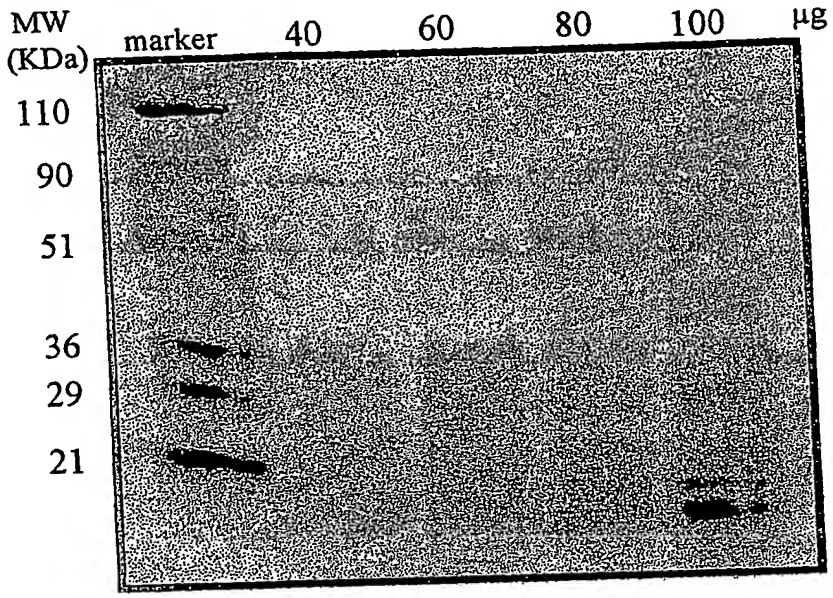
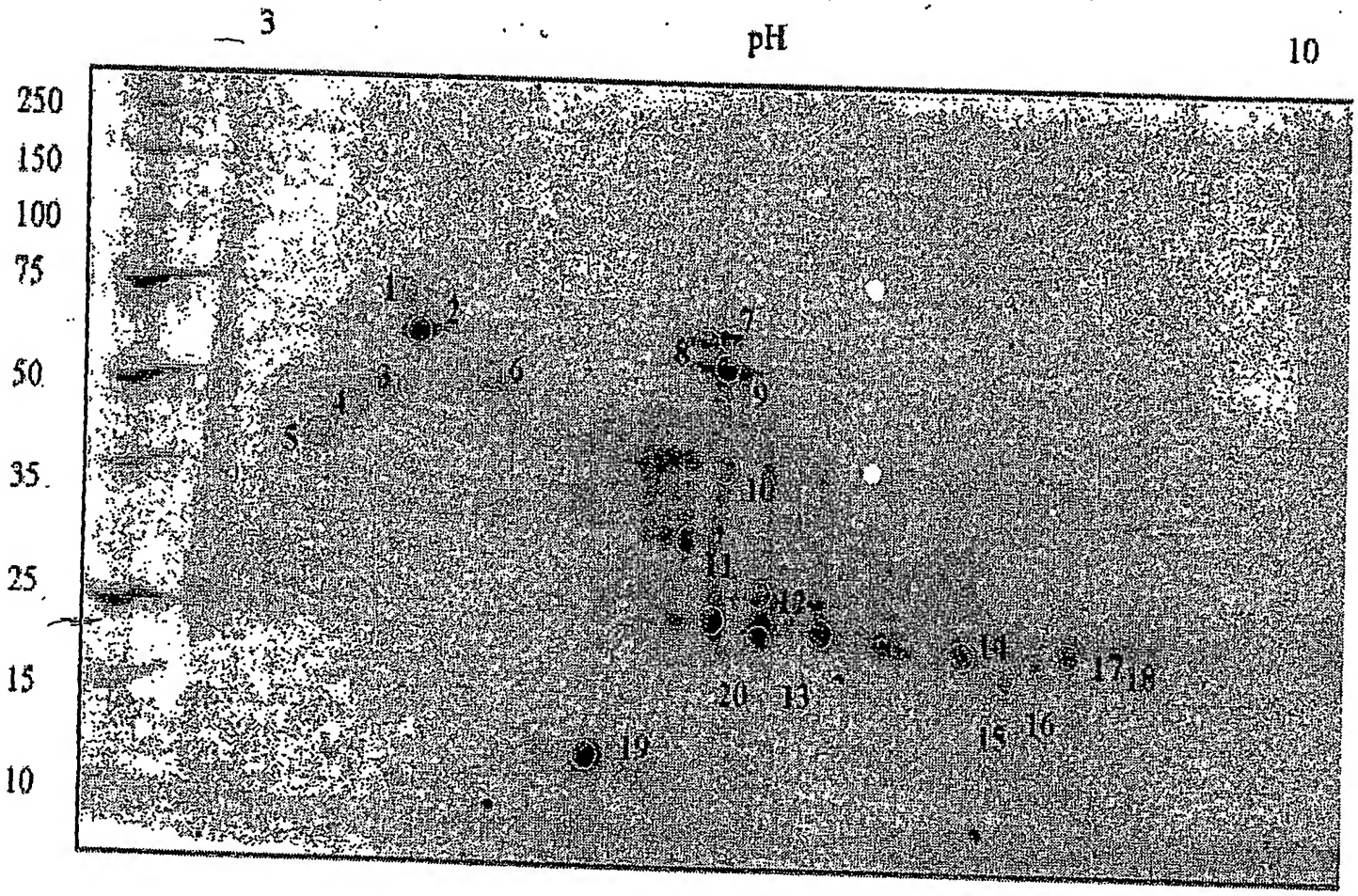


Figure 3



. CBT protein sample, pH3-10 gradient: Proteins annotated for MALDI-MS

FIGURE 4

1
Tentative
NCF1_BOVIN
Neutrophil cytosol factor 1
Molecular weight: 45346
Matches: 5
MOWSE Score: 1.6076937e+003
Likelihood: 1.96e+003
Coverage: 14.80 %
Matching peptides:
MW Delta Start End Sequence
841.4657 -88.56 120 126 (K)VRPDD LK(L)
886.4760 -21.99 127 134 (K)LPTDS QVK(K)
886.4508 -50.37 283 291 (K)AGQDV AQAK(S)
* 1164.5822 0.02 328 336 (R)NSVRF MQQR(R)
1730.7934 -0.01 56 70 (K)EMFPI EAGDI NPENR(I)
1891.9197 23.26 170 188 (K)GSSSQ MALAT GDVVD VVEK(N)
OR
Tentative
ALBU_BOVIN
Bovine Serum Albumin
Molecular weight: 69294
Matches: 5
MOWSE Score: 7.6716406e+001
Likelihood: 1.88e+003
Coverage: 8.73 %
Matching peptides:
MW Delta Start End Sequence
711.3664 59.75 29 34 (K)SEIAH R(F)
* 959.5400 -35.02 210 218 (R)EKVLA SSAR(Q)
* 1000.5818 -23.99 233 241 (R)ALKAW SVAR(L)
1385.6133 -28.71 286 297 (K)YICDN QDTIS SK(L)
* 1961.9404 26.67 139 155 (K)LKPDP NTLCD EFKAD EK(K)
2

Tentative

Score: 0.19, 6 matching peptides: P35445 (COMP_BOVIN) pI: undefined, Mw: undefined

Cartilage oligomeric matrix protein (COMP) (Fragment). - Bos taurus (Bovine).

user mass matching [Delta] #MC modification positionpeptide

mass (ppm) mass

887.4908 887.4404 -56.88 0 Cys_PAM: 10 8-14 DNCPLVR

1181.5063 1181.4463 -50.84 0 2xCys_PAM 26-34 WGDACDNCR

1226.6927 1226.631 -50.35 1 Cys_PAM: 69 62-71 IRNPVDNCPK

1337.5306 1337.491 -29.64 0 Cys_PAM: 53 50-61 GDACDDDIDGDR

Page 3

1370.707 1370.6369 -51.19 0 168-179 LVPNPGQEDMDR

1386.6544 1386.6318 -16.32 0 MSO: 177 168-179 LVPNPGQEDMDR

11.4% of sequence covered:

3

No Good Match

4

No Good Match

5

No Good Match

6

No Good Match

7

ALBU_BOVIN

Bovine Serum Albumin

Molecular weight: 69294

Matches: 11

MOWSE Score: 9.5664269e+006

Likelihood: 4.27e+003

Coverage: 17.96 %

Matching peptides:

MW Delta Start End Sequence

926.4862 -137.94 161 167 (K)YLYEI AR(R)

1162.6234 -74.58 66 75 (K)LVNEL TEFAK(T)

1282.7033 -96.76 361 371 (R)HPEYA VSVLL R(L)

1304.7088 -90.40 402 412 (K)HLVDE PQNLI K(Q)
* 1438.8045 -82.02 360 371 (R)RHPEY AVSVL LR(L)
1478.7881 -92.19 421 433 (K)LGEYG FQNAL IVR(Y)
1510.8355 -83.71 438 451 (K)VPQVS TPTLV EVSR(S)
1566.7354 -87.40 347 359 (K)DAFLG SFLYE YSR(R)
* 1638.9304 -66.09 437 451 (R)KVPQV STPTL VEVSR(S)
1414.6802 -38.28 569 580 (K)TVMEN FVAFV DK(C)
+ Methionine Sulfoxide
1893.9294 -61.74 508 523 (R)RPCFS ALTPD ETYVP K(A)
+ Cysteine acrylamide
Page 4
8
ALBU_BOVIN
Bovine Serum Albumin
Molecular weight: 69294
Matches: 13
MOWSE Score: 7.6034479e+007
Likelihood: 1.17e+004
Coverage: 21.42 %
Matching peptides:
MW Delta Start End Sequence
926.4862 -72.22 161 167 (K)YLYEI AR(R)
1162.6234 -28.39 66 75 (K)LVNEL TEFAK(T)
1282.7033 -47.50 361 371 (R)HPEYA VSVLL R(L)
1304.7088 -31.77 402 412 (K)HLVDE PQNLI K(Q)
* 1438.8045 -39.49 360 371 (R)RHPEY AVSVL LR(L)
1478.7881 -32.08 421 433 (K)LGEYG FQNAL IVR(Y)
1510.8355 -19.52 438 451 (K)VPQVS TPTLV EVSR(S)
1518.7388 -25.79 139 151 (K)LKPDP NTLCD EFK(A)
1566.7354 -34.55 347 359 (K)DAFLG SFLYE YSR(R)
* 1638.9304 -19.36 437 451 (R)KVPQV STPTL VEVSR(S)
* 988.5488 24.47 221 228 (R)LRCAS IQK(F)
+ Cysteine acrylamide
1414.6802 8.58 569 580 (K)TVMEN FVAFV DK(C)
+ Methionine Sulfoxide
1893.9294 -10.63 508 523 (R)RPCFS ALTPD ETYVP K(A)

+ Cysteine acrylamide

9

NCF1_BOVIN

10

Q95L50

Type IX collagen alpha 1 chain

Molecular weight: 20907

Matches: 5

MOWSE Score: 3.7725965e+003

Likelihood: 5.46e+003

Coverage: 28.34 %

Matching peptides:

MW Delta Start End Sequence

933.4668 -63.69 61 68 (K)LGNNV DFR(I)

1051.6390 -43.95 173 181 (R)IESLP IKPR(G)

2183.0687 42.58 73 90 (R)HLYPN GLPEE YSFLT TFR(M)

832.4476 -30.07 155 161 (K)IMIGV ER(S)

+ Methionine Sulfoxide

1282.5976 -22.54 162 172 (R)SSATL FVDCN R(I)

+ Cysteine acrylamide

11

ALBU_BOVIN

Bovine Serum Albumin

Molecular weight: 69294

Matches: 5

MOWSE Score: 1.1175735e+003

Likelihood: 7.26e+002

Coverage: 10.21 %

Matching peptides:

MW Delta Start End Sequence

926.4862 -156.39 161 167 (K)YLYEI AR(R)

1282.7033 -36.27 361 371 (R)HPEYA VSVLL R(L)

1566.7354 -84.65 347 359 (K)DAFLG SFLYE YSR(R)

* 1887.9876 -6.32 89 105 (K)SLHTL FGDEL CKVAS LR(E)

1887.9195 -42.39 169 183 (R)HPYFY APELL YYANK(Y)

1790.7021 -70.93 267 280 (K)ECCHG DLLEC ADDR(A)
+ Cysteine acrylamide
+ Cysteine acrylamide
+ Cysteine acrylamide
12
ALBU_BOVIN
Bovine Serum Albumin
Molecular weight: 69294
Matches: 16
MOWSE Score: 3.5119435e+010
Likelihood: 1.04e+004
Coverage: 28.17 %
Matching peptides:
MW Delta Start End Sequence
926.4862 -106.87 161 167 (K)YLYEI AR(R)
1282.7033 28.51 361 371 (R)HPEYA VSVLL R(L)
1304.7088 -33.76 402 412 (K)HLVDE PQNLI K(Q)
1478.7881 -58.18 421 433 (K)LGEYG FQNAL IVR(Y)
1510.8355 -34.08 438 451 (K)VPQVS TPTLV EVSR(S)
* 1638.9304 -30.89 437 451 (R)KVPQV STPTL VEVSR(S)
* 1737.8032 -39.00 387 401 (K)DDPHA CYSTV FDKLK(H)
1120.5223 -76.32 588 597 (K)EACFA VEGPK(L)
+ Cysteine acrylamide
1165.5220 -96.70 499 507 (K)CCTES LVNR(R)
+ Cysteine acrylamide
+ Cysteine acrylamide
1193.5169 -102.89 460 468 (R)CCTKP ESER(M)
+ Cysteine acrylamide
+ Cysteine acrylamide
1414.6802 16.07 569 580 (K)TVMEN FVAFV DK(C)
+ Methionine Sulfoxide
1567.6613 9.82 387 399 (K)DDPHA CYSTV FDK(L)
+ Cysteine acrylamide
1589.7759 24.36 139 151 (K)LKPDP NTLCD EFK(A)
+ Cysteine acrylamide

Page 6
1753.8379 17.95 469 482 (R)MPCTE DYLSL ILNR(L)
+ Methionine Sulfoxide
+ Cysteine acrylamide
1893.9294 -3.13 508 523 (R)RPCFS ALTPD ETYVP K(A)
+ Cysteine acrylamide
1920.9291 10.74 529 544 (K)LFTFH ADICT LPDTE K(Q)
+ Cysteine acrylamide
13
Q95L50
Type IX collagen alpha 1 chain
Molecular weight: 20907
Matches: 9
MOWSE Score: 1.6928014e+007
Likelihood: 1.54e+004
Coverage: 62.03 %
Matching peptides:
MW Delta Start End Sequence
816.4018 -95.42 124 130 (K)SVSFS YK(G)
816.4527 -32.98 155 161 (K)IMIGV ER(S)
933.4668 -51.91 61 68 (K)LGNNV DFR(I)
1051.6390 -49.75 173 181 (R)IESLP IKPR(G)
1570.7528 -9.01 99 111 (K)HWSIW QIQDS SGK(E)
2148.0739 -6.99 21 39 (R)IGQDD LPGFD LISQF QIDK(A)
2183.0687 -12.48 73 90 (R)HLYPN GLPEE YSFLT TFR(M)
2618.2765 -0.04 131 154 (K)GLDGS LQTAA FSNLP SLFDS QWHK(I)
832.4476 -3.52 155 161 (K)IMIGV ER(S)
+ Methionine Sulfoxide
1282.5976 -44.29 162 172 (R)SSATL FVDCN R(I)
+ Cysteine acrylamide
14
Q95L50
Type IX collagen alpha 1 chain
Molecular weight: 20907
Matches: 9
MOWSE Score: 1.6928014e+00

Likelihood: 8.48e+003
Coverage: 62.03 %
Matching peptides:
MW Delta Start End Sequence
816.4018 -156.04 124 130 (K)SVSFS YK(G)
816.4527 -93.60 155 161 (K)IMIGV ER(S)
933.4668 -138.67 61 68 (K)LGNNV DFR(I)
1051.6390 -126.57 173 181 (R)IESLP IKPR(G)
1570.7528 -78.20 99 111 (K)HWSIW QIQDS SGK(E)
2148.0739 -53.45 21 39 (R)IGQDD LPGFD LISQF QIDK(A)
Page 7
2183.0687 -61.45 73 90 (R)HLYPN GLPEE YSFLT TFR(M)
2618.2765 -0.10 131 154 (K)GLDGS LQTAA FSNLP SLFDS QWHK(I)
832.4476 -105.98 155 161 (K)IMIGV ER(S)
+ Methionine Sulfoxide
1282.5976 -119.59 162 172 (R)SSATL FVDCN R(I)
+ Cysteine acrylamide
15
Q95L50
Type IX collagen alpha 1 chain
Molecular weight: 20907
Matches: 7
MOWSE Score: 5.0424749e+005
Likelihood: 8.67e+003
Coverage: 48.13 %
Matching peptides:
MW Delta Start End Sequence
816.4018 103.26 124 130 (K)SVSFS YK(G)
816.4527 165.71 155 161 (K)IMIGV ER(S)
933.4668 13.11 61 68 (K)LGNNV DFR(I)
1051.6390 5.50 173 181 (R)IESLP IKPR(G)
1570.7528 -0.05 99 111 (K)HWSIW QIQDS SGK(E)
2183.0687 -17.84 73 90 (R)HLYPN GLPEE YSFLT TFR(M)
2618.2765 0.11 131 154 (K)GLDGS LQTAA FSNLP SLFDS QWHK(I)
1282.5976 -29.95 162 172 (R)SSATL FVDCN R(I)
+ Cysteine acrylamide

16

Q95L50

Type IX collagen alpha 1 chain

Molecular weight: 20907

Matches: 5

MOWSE Score: 1.6468835e+004

Likelihood: 8.25e+003

Coverage: 34.76 %

Matching peptides:

MW Delta Start End Sequence

933.4668 35.82 61 68 (K)LGNNV DFR(I)

1051.6390 30.60 173 181 (R)IESLP IKPR(G)

2148.0739 0.08 21 39 (R)IGQDD LPGFD LISQF QIDK(A)

2183.0687 -1.95 73 90 (R)HLYPN GLPEE YSFLT TFR(M)

1282.5976 -7.80 162 172 (R)SSATL FVDCN R(I)

+ Cysteine acrylamide

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17

Q95L50

Type IX collagen alpha 1 chain

Molecular weight: 20907

Matches: 8

MOWSE Score: 9.0085540e+005

Likelihood: 5.86e+003

Coverage: 49.20 %

Matching peptides:

MW Delta Start End Sequence

816.4018 -137.06 124 130 (K)SVSFS YK(G)

816.4527 -74.62 155 161 (K)IMIGV ER(S)

933.4668 -125.07 61 68 (K)LGNNV DFR(I)

1051.6390 -117.92 173 181 (R)IESLP IKPR(G)

1570.7528 -50.26 99 111 (K)HWSIW QIQDS SGK(E)

2148.0739 -4.71 21 39 (R)IGQDD LPGFD LISQF QIDK(A)

2183.0687 -24.99 73 90 (R)HLYPN GLPEE YSFLT TFR(M)

832.4476 -86.04 155 161 (K)IMIGV ER(S)

+ Methionine Sulfoxide

1282.5976 -97.22 162 172 (R)SSATL FVDCN R(I)
+ Cysteine acrylamide
18
Q95L50
Type IX collagen alpha 1 chain
Molecular weight: 20907
Matches: 5
MOWSE Score: 1.6468835e+004
Likelihood: 2.56e+003
Coverage: 34.76 %
Matching peptides:
MW Delta Start End Sequence
933.4668 -122.28 61 68 (K)LGNNV DFR(I)
1051.6390 -107.17 173 181 (R)IESLP IKPR(G)
2148.0739 0.02 21 39 (R)IGQDD LPGFD LISQF QIDK(A)
2183.0687 -6.21 73 90 (R)HLYPN GLPEE YSFLT TFR(M)
1282.5976 -90.21 162 172 (R)SSATL FVDCN R(I)
+ Cysteine acrylamide
19
OBP_BOVIN
Odorant-binding protein
Molecular weight: 18503
Matches: 7
MOWSE Score: 4.8550116e+005
Likelihood: 2.71e+004
Coverage: 50.94 %
Page 9
Matching peptides:
MW Delta Start End Sequence
959.4825 -4.49 30 37 (K)IQENG PFR(T)
993.4655 32.50 42 49 (R)ELVFD DEK(G)
1161.5706 1.66 50 59 (K)GTVDF YFSVK(R)
1207.6085 18.62 19 29 (R)TVYIG STNPE K(I)
1359.7259 -11.18 97 108 (R)THLVA HNINV DK(H)
1788.8067 1.86 145 159 (K)NVVNF LENED HPHPE(-)
1947.8486 2.44 74 90 (K)QDDGT YVADY EGQNV FK(I)

20

Q95L50

Type IX collagen alpha 1 chain

Molecular weight: 20907

Matches: 8

MOWSE Score: 9.0085540e+005

Likelihood: 6.84e+003

Coverage: 49.20 %

Matching peptides:

MW Delta Start End Sequence

816.4018 -121.38 124 130 (K)SVSFS YK(G)

816.4527 -58.95 155 161 (K)IMIGV ER(S)

933.4668 -106.00 61 68 (K)LGNNV DFR(I)

1051.6390 -108.98 173 181 (R)IESLP IKPR(G)

1570.7528 -54.97 99 111 (K)HWSIW QIQDS SGK(E)

2148.0739 -18.35 21 39 (R)IGQDD LPGFD LISQF QIDK(A)

2183.0687 -25.77 73 90 (R)HLYPN GLPEE YSFLT TFR(M)

832.4476 -81.84 155 161 (K)IMIGV ER(S)

+ Methionine Sulfoxide

1282.5976 -93.79 162 172 (R)SSATL FVDCN R(I)

+ Cysteine acrylamide

21

Q95L50

Type IX collagen alpha 1 chain

Molecular weight: 20907

Matches: 8

MOWSE Score: 9.0085540e+005

Likelihood: 6.26e+003

Coverage: 49.20 %

Matching peptides:

MW Delta Start End Sequence

816.4018 -133.87 124 130 (K)SVSFS YK(G)

816.4527 -71.44 155 161 (K)IMIGV ER(S)

933.4668 -113.93 61 68 (K)LGNNV DFR(I)

1051.6390 -115.35 173 181 (R)IESLP IKPR(G)

1570.7528 -60.83 99 111 (K)HWSIW QIQDS SGK(E)

2148.0739 -9.04 21 39 (R)IGQDD LPGFD LISQF QIDK(A)

2183.0687 -23.66 73 90 (R)HLYPN GLPEE YSFLT TFR(M)

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832.4476 -68.62 155 161 (K)IMIGV ER(S)

+ Methionine Sulfoxide

1282.5976 -93.25 162 172 (R)SSATL FVDCN R(I)

+ Cysteine acrylamide

Fig. 7: Physical Characteristics of Protein fragments found in Calcium Peptacan after ion-exchange treatment and 2D electrophoresis.

Fragment ID#	Estimated pI	Estimated Molecular Weight
	Approximately:	Approximately:
1	4.0	74kDa
2	4,1	65kDa
3	3.9	50kDa
	(this band has now been shown to correspond to fragments from 2 or 3 proteins identified as bovine COMP and either bovine alpha-1 antiprotease inhibitor or Endopin-1)	
4	3.7	45kDa
5	3.4	40kDa
6	4.6	50kDa
7	6.3	67kDa
8	6.1	65kDa
9	6.3	60kDa
10	6.3	38kDa
11	6.0	30kDa
12	6.4	25kDa
13	6.8	22kDa
14	7.8	20kDa
15	8.2	18kDa
16	8.3	19kDa
17	8.6	20kDa
18	9.1	19kDa
19	5.3	12kDa
20	6.2	22kDa
21	7.2	21kDa

Figure 5

dgvlnekdnc plvrnpdqrn tdgdkwgdac dncrsqkndd qkdtdkdgrg dacdddidgd rirnpvdncp
kvpnsdqkdt dgdgvgdacd ncpqksnadq rdvdhdfvgd acdsdqdqdg dghqdskdnc ptvpnsaqqd
sdhdgqgdac ddddndgvp dsrdncrlvp npgqedmdrd gvgdacqgdf dadkvvdkid vcpenaevtl
tdfrafqtvv ldpegdaqid pnwvvlnqgm eivqtmnsdp glcvgytafn gvdfegpfhv ntatdddyag
fifgyhhsss fyvvmwkqme qtywqanpfr avaepgiqlk avksstgpge qlrnalwhtg dtasqvrllw
kdprnvgwkd ktsyrwflqh rpqvgyirvr fyegpelvad snvildttmr ggrlgvfcfs qeniiwanlr
yrcndtiped yeaqrllqa

Figure 6

mawtardrga lgllllglcl caaqrgppge qgppgasgpp gvpgidgidg drgpkgppgp pgpagepgkp
gapgkpgtpg adgltgpdgs pgsigskgqk gepgvpgsrg fpgrgipgpp gppgtaglpg elgrvgpvgd
pgrrgppgpp gppgprgtig fhdgdplcpn acppgrsgyp glpgmrghkg akgeigepgr qghkgeegdq
gelgevgaqg ppgaqglrgi tgivgdkgek gargldgepg pqglpgapgd qgqrgppgea gpkgdrgaeg
argipglpgp kgdtglpgvd grdgipgmpg tkgepgkpgp pgdaglqglp gvpgipgakg vagekgstga
pgkpgqmgns gkpgqqgppg evgprgpqgl pgsrgelgpv gspglpgklg slgspglpgl pgppglpgmk
gdrgvvgepg pkgeqgasge egeagergel gdiglpgpkg sagnpgepgl rgpegsrglp gvegprgppg
prgvqgeqga tglpgvqgpp graptdqhik qvcmrviqeh faemaaslkr pdsgatglpg rpgppgppgp
pgengfpgqm girglpgikg ppgalglrgp kgdlgekger gppgrgpngl pgaiglpgdp gpasygkngr
dgergppgla gipgvpgppg ppglpgfcep asctmqagqr afnkgpdp

TABLE 2:Results Rat CIA-Prophylactic Treatment
Treatments (Rx) given for 15 days (0-14)
No Rx after Day 14 - rebound measured on Day 20

		Mean arthritis scores			Signs of arthritis Day 15			Signs of arthritis Day 20		
Rx n=4	Dose mg/kg	Day 13	Day 15	Day 28 no drug	R/paw swell	F/paw swell	Weight change	R/paw swell	F/paw swell	Weight change
None	—	1.1	1.9	N/A	1.02	2.9	+50g	N/A	N/A	N/A
INR-195	200	0.2	0.5	3.0	0.26	0.3	+46g	1.23	2.3	+0.2g
INR-195	20	0.3	0.2	1.2	0.14	0.7	+37g	0.38	2.0	+10g
Flax-seed oil	1800	1.7	>2.5	N/A	1.22	3.2	+47g	N/A	N/A	N/A
Aurothio-malate	6.3*	1.5	>2.5	3.0	1.13	3.0	+35g	1.0	3.0	0

*dosed every second day, N/A = not available

Figure 8

TABLE3:Results Rat CIA Therapeutic Treatment

treatment for 3 days only initiated either Day 15 or Day 17

			Mean reduction in signs of arthritis after 3 days treatment				
Rx n=3	Dose mg/kg	Days	Rear Paw Swelling	Tail Swelling	Fore Paw Inflammation	Weight Change	Arthritis Score
INR-195	200	15-18	0.53	0.11	1.1	-11gm**	+
INR-195	200	17-20	0.65	0.37	+	0	0.8+
Meloxic-am*	5	15-18	0	0.23	0	0.05+	0.5+
Meloxic-am*	10	15-18	0.62	0.08	0.5+	-.04**	0.5+

*human recommended anti-inflammatory dose 7.5mg per day
**negative values = weight gain

Figure 9

TABLE 4: GASTROTOXICITY ON DAY 20 IN RAT CIA WITH IBUPROFEN (50 mg/kg) FOLLOWED BY DRUG TREATMENT

Treatment mg/kg	Period days	Mean Score*	# Rats
None	—	25	7
INR195 (200)	15	9	3
INR195 (20)	15	4	3
INR195 (200)	3 day later	7	4
Aurothiomalate**	15	14	2
Normal Rats	—	2	5

*Mean Score = number of haemorrhagic lesions
**Given 6.3 mg/kg s.c. on alternate days

Figure 10